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PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Atty. Dkt. No.: MYOG:004DIV1

Prior Application Examiner:
J. Souaya

BOX PATENT APPLICATION

Assistant Commissioner for Patents
Washington, D.C. 20231

Classification Designation:

Prior Group Art Unit: 1655

**REQUEST FOR FILING DIVISIONAL APPLICATION
UNDER 37 C.F.R. § 1.53(b)**

This is a request for filing a divisional application under Rule 53(b) (37 C.F.R. § 1.53(b)) of co-pending prior application Serial No. 09/016,075 filed January 30, 1998, entitled "DIAGNOSIS AND TREATMENT OF MYOCARDIAL FAILURE," by inventors Michael R.BRISTOW, Leslie A. LEINWAND, Wayne MINOBE, and Koichi NAKAO.

1. Enclosed is a copy of the prior application Serial No. 09/016,075 as originally filed, including specification, claims, drawings, and declaration. The undersigned hereby verifies that the attached papers are a true copy of the prior application as originally filed and identified above, that no amendments (if any) referred to in the declaration filed to complete the prior application introduced new matter therein, and further that this statement was made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment or both, under Section 1001 of Title 18 of the United States Code, and that such

willful false statement may jeopardize the validity of the application or any patent issuing thereon.

(a) The inventorship is the same as prior Application Serial No. 09/016,075.

(b) Deletion of inventor(s). Signed statement attached deleting inventor(s) named in the prior application, see 37 C.F.R. § 1.63(d)(2) and 1.33(b).

(c) Priority of foreign patent application number , filed in is claimed under 35 U.S.C. § 119(a)-(e). The certified copy:
 is enclosed.
 has been filed in the prior Application Serial No.

2. The Assistant Commissioner is requested to grant Applicant a filing date in accordance with Rule 1.53, and supply Applicant with a Notice of Missing Parts in due course, in accordance with the provisions of Rule 1.53(f).

3. Enclosed is a check in the amount of \$345.00 to cover the filing fee as calculated below and the fee for any new claims added in the Preliminary Amendment referred to in Part No. 9 below.

CLAIMS AS FILED IN THE PRIOR APPLICATION
LESS CLAIMS CANCELED BELOW

FOR	NUMBER FILED	NUMBER EXTRA	RATE	FEE
Basic Fee -----				\$345.00
Total Claims	7 - 20 =	0 X	\$9.00 =	\$0.00
Independent Claims	1 - 3 =	0 X	\$39.00 =	\$0.00
Multiple Dependent Claim(s) -----				\$-0-.00
TOTAL FILING FEES:				\$345.00

4. Applicant is entitled to Small Entity Status for this application.

(a) A small entity statement is enclosed.

(b) A small entity statement was filed in the prior nonprovisional application and such status is still proper and desired.

(c) Small entity status is no longer claimed.

5. If the check is missing or insufficient, the Assistant Commissioner is hereby authorized to charge any fees under 37 C.F.R. §§ 1.16 to 1.21 which may be required for any reason relating to this application, or credit any overpayment to Fulbright & Jaworski Account No.: 50-1212/10014732/01973.

6. Enclosed is a copy of the current Power of Attorney in the prior application.

7. Address all future communications to:

Steven L. Highlander
FULBRIGHT & JAWORSKI, L.L.P.
600 Congress Avenue, Suite 2400
Austin, Texas 78701
(512) 418-3184

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- 8. The prior application is presently assigned to UNIVERSITY TECHNOLOGY CORPORATION.
- 9. Enclosed is a preliminary amendment. Any additional fees incurred by this amendment are included in the check at No. 3 above and said fee has been calculated after calculation of claims and after amendment of claims by the preliminary amendment.
- 10. Cancel in this application claims 1-16 and 24-26 of the prior application before calculating the filing fee. (At least one original independent claim must be retained).
- 11. Amend the specification by inserting before the first line the sentence: --This is a divisional of co-pending application Serial No. 09/016,075 filed January 30, 1998, which claims priority to U.S. Provisional applications Serial Nos. 60/038,911, filed February 26, 1997, and 60/036,987, filed January 30, 1997. --
- 12. Enclosed are formal drawings.
- 13. An Information Disclosure Statement (IDS) is enclosed.
 - (a) PTO-1449.
 - (b) Copies of IDS citations.
- 14. Transfer the sequence information, including the computer readable form previously submitted in the parent application, Serial No. 09/016,075 filed January 30, 1998, for use in this application. **Under 37 C.F.R. § 1.821(e), Applicant states that the paper copy of the sequence listing in this application is identical to the computer readable copy in parent application Serial No. 09/016,075 filed January 30, 1998.** **Under 37 C.F.R. § 1.821(f), Applicant also states that the**

information recorded in computer readable form is identical to the written sequence listing.

- 15. Other: copy of Request for Extension of Time filed in parent application concurrently herewith.
- 16. Return Receipt Postcard (should be specifically itemized).

Respectfully submitted,



Steven L. Highlander
Reg. No. 37,642
Attorney for Applicant

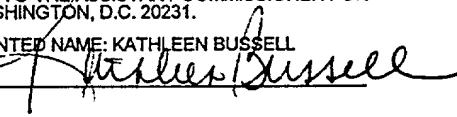
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Case No. 3595-6

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE
APPLICATION FOR LETTERS PATENT

TITLE:

DIAGNOSIS AND TREATMENT OF
MYOCARDIAL FAILURE

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0009247-3241857573

DIAGNOSIS AND TREATMENT OF MYOCARDIAL FAILURE

Benefit of provisional applications 60/036,987, filed January 30, 1997, and 60/038,911, filed February 26, 1997, is
5 hereby claimed.

This invention was made with government support under NIH grants 5R37 HL 50530-04, RO1 HL 48013, and GCRC-CAP 5 MO 1 RR00051. The government has certain rights in the invention.

10 FIELD OF THE INVENTION

The present invention relates to a method of diagnosing myocardial failure in humans by quantitating the expression of α -myosin heavy chain (α -MHC), β -myosin heavy chain (β -MHC), or both in a sample of myocardial tissue from a ventricle of the heart. The invention further relates to a method of treating myocardial failure in aging humans or humans suffering from heart failure by increasing the expression of α -MHC in myocardial tissue.

20 BACKGROUND OF THE INVENTION

Heart failure is a pathophysiological state in which the heart fails to pump blood at a rate commensurate with the requirements of the metabolizing tissues of the body. It is caused in most cases (about 95% of cases) by myocardial failure.

The contractile proteins of the heart lie within the muscle cells (myocytes), which constitute about 75% of the total volume of the myocardium. The two major contractile proteins are the thin actin filament and the thick myosin filament. Each myosin filament contains two heavy chains and four light chains. The bodies of the heavy chains are intertwined, and each heavy chain ends in a head. Each lobe

of the bilobed myosin head has an ATP-binding pocket, which has in close proximity the myosin ATPase activity that breaks down ATP to its products.

The velocity of cardiac muscle contraction is controlled
5 by the degree of ATPase activity in the head regions of the myosin molecules. The major determinant of myosin ATPase activity and, therefore, of the speed of muscle contraction, is the relative amounts of the two myosin heavy chain isomers, alpha and beta (α -MHC and β -MHC). The α -MHC isoform has
10 approximately four times more enzymatic activity than the β -MHC isoform and, consequently, the velocity of cardiac muscle shortening is related to the relative percentages of each isoform. For example, adult rodent ventricular myocardium has approximately 80-90% α -MHC and only 10-20% β -MHC, which
15 explains why its myosin ATPase activity is 3-4 times greater than bovine ventricular myocardium, which contains 80-90% β -MHC.

When ventricular myocardial hypertrophy or heart failure is created in rodent models, a change occurs in the expression
20 of MHC isoforms, with α -MHC decreasing and β -MHC becoming the dominant isoform. These "isoform switches" then reduce the contractility of the hypertrophied rodent ventricle, ultimately leading to myocardial failure. This pattern of altered gene expression has been referred to as reversion to
25 a "fetal" pattern because, during fetal and early neonatal development, β -MHC also dominates in rodent ventricular myocardium.

Although human atrial myocardium may undergo similar isoform switches with hypertrophy or failure, human
30 ventricular myocardium, the basis for the majority of cases of heart failure (greater than 90% of cases), has not been thought to exhibit this pattern. This is because several studies which examined this issue in autopsy cases did not

find biologically significant expression of the α -MHC isoform in putatively normal hearts. Since there was thought to be no significant expression of α -MHC in normal hearts, a downregulation in α -MHC was not thought to be a possible basis
5 for myocardial failure in humans. There has been one report that the amount of α -MHC, although extremely small to begin with, is reduced in failing human myocardium. Bouvagnet et al., *Basic Res. Cardiol.*, 84, 91-102 (1989). There have also been conflicting reports about the presence and amounts of α -
10 MHC and β -MHC messenger RNA (mRNA) in normal and failing human myocardium. Cf. Arai et al., *Circ. Res.*, 72, 463 (1993) with Lowes et al., *J. Invest. Med.*, 43, 316A (1995). However, as of 1997, those skilled in the art still considered it unlikely
15 that a shift in myosin isoforms occurred in human myocardial failure. See Colucci and Braunwald, in *Heart Disease: A Textbook of Cardiovascular Medicine* (Braunwald ed., 5th ed., 1997), Chapter 13 at page 406. Indeed, MHC gene expression is considered to be a classical example of species variation in the expression of genes, and data on MHC gene expression in
20 human disease states cannot be extrapolated from animal studies.

It has been shown that myocardial function declines with age in animals. Cellular and molecular mechanisms that account for age-associated changes in myocardial performance
25 have been studied largely in rodents. Among other changes, marked shifts in MHC occur in rodents, i.e., the β isoform becomes predominant in senescent rats (85% β versus 15% α). Steady-state mRNA levels for α -MHC and β -MHC parallel the age-associated change in the MHC proteins. The myosin ATPase
30 activity declines with the decline in α -MHC content, and the altered cellular profile results in a contraction that exhibits a reduced velocity and a prolonged time course. No

similar studies are known to have been made in humans and, as noted above, data on MHC gene expression from animal studies cannot be extrapolated to humans.

For a detailed discussion of heart failure, MHC gene expression, and age-associated changes in cardiac performance,
5 see *Heart Disease: A Textbook of Cardiovascular Medicine* (Braunwald ed., 5th ed., 1997).

SUMMARY OF THE INVENTION

10 The invention provides a method of diagnosing myocardial failure in a human. The method comprises obtaining a sample of myocardial tissue from a ventricle of the heart of the human. The expression of α -myosin heavy chain (α -MHC), β -myosin heavy chain (β -MHC), or both in the sample is
15 quantitated. Then, it is determined by statistical analysis if the expression of α -MHC, β -MHC, or both in the sample is significantly different than their expression in normal human ventricular myocardial tissue.

The invention also provides a kit for diagnosing
20 myocardial failure in a human. The kit comprises a container holding at least one nucleic acid molecule that hybridizes to DNA or RNA coding for α -MHC, β -MHC or both.

The invention further provides a method of treating myocardial failure in a human. The method comprises
25 administering an effective amount of an agent that directly causes an increase in the quantity of α -MHC in the myocardial tissue of the heart.

Finally, the invention provides a method of quantitating the expression of a first protein relative to the expression
30 of a second protein or to the total expression of the first and second proteins. The method comprises obtaining a sample of cells or tissue expressing the first protein and the

second protein, extracting RNA from the cells or tissue, preparing cDNA from the RNA, amplifying the cDNA coding for the first and second proteins by polymerase chain reaction using primers that hybridize to cDNA coding for the first 5 protein, the second protein or both, and quantitating the amplified PCR products.

BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-B: Graphs showing the effect of a four-hour 10 (Figure 1A) or a twelve-hour (Figure 1B) incubation with 1 nM triiodothyronine (T3) on peak systolic tension response to isoproterenol in isolated right ventricular trabeculae removed from end-stage failing human hearts.

15 DETAILED DESCRIPTION OF THE PRESENTLY PREFERRED EMBODIMENTS OF THE INVENTION

The invention provides a method of diagnosing myocardial failure in a human. "Myocardial failure" means a defect in myocardial contraction or an abnormality of myocardial 20 function. As noted above, myocardial failure is the cause of most cases (greater than 90% of cases) of heart failure, and it occurs in aging hearts.

The diagnostic method of the invention comprises quantitating the expression of α -MHC, β -MHC, or both in a 25 sample of myocardial tissue taken from ventricular myocardium. It has been found that the expression of α -MHC and β -MHC in the left ventricle is similar to that in the right ventricle when the function of the two ventricles is similar (see Example 3 below). Since myocardial failure occurs in both 30 ventricles in aging and in most cases of heart failure, it is expected that, in most cases, either ventricle can be used as a sampling site. Although, the left ventricle is of primary importance because it is responsible for pumping blood to all

of the tissues of the body, except the lungs, the right ventricle will generally be used as the sampling site because of ease of sampling. In cases where only the right ventricle is suspected of failing (e.g., in primary pulmonary hypertension), then the right ventricle must be sampled. Similarly, in cases where only the left ventricle is suspected of failing (e.g., subsequent to myocardial infarction in the left ventricle), then the left ventricle must be sampled.

The sample of ventricle tissue to be tested is preferably procured from a living patient by endomyocardial biopsy, a technique well known in the art. See Wynne and Braunwald, in *Heart Disease: A Textbook of Cardiovascular Medicine* (Braunwald ed., 5th ed., 1997), Chapter 41 at pages 1404-1406. The tissue sample may also be obtained from hearts removed at autopsy or prior to transplantation of replacement hearts.

The diagnostic method of the invention may be used to make a diagnosis of a patient's condition. The method may also be used to assess the effectiveness of the drugs or other measures used to treat a patient diagnosed as suffering from myocardial failure.

The expression of α -MHC, β -MHC, or both in the test sample must be compared with their expression in normal ventricular myocardial tissue from age-matched controls. The normal tissue should be from the same ventricle as the test sample. The comparison is made using standard methods of statistical analysis well known in the art.

The normal tissue samples can be obtained from hearts removed from individuals dying of non-cardiac causes, primarily accidental deaths and homicides. For instance, samples from explanted hearts intended, but not used, for transplantation can be used. Alternatively, myocardial biopsy samples from living donors can be used. For instance,

myocardial biopsy samples from patients suspected of having heart disease, but subsequently determined to be normal, can be used (see Example 1). Also, the normal values set forth in the examples below may be used for comparison purposes.

5 "Normal" and "nonfailing" are used herein interchangeably. Both are intended to mean that the hearts show no signs of myocardial failure and appear to function normally.

A diagnosis of myocardial failure can be made if the
10 statistical analysis shows: (1) that the expression of α -MHC in the test sample is significantly lower than it is in the age-matched normal tissue; (2) that the expression of β -MHC in the test sample is significantly higher than it is in the age-matched normal tissue; (3) both of these; or (4) the ratio of
15 α -MHC expression to β -MHC expression or total MHC expression (α -MHC/ β -MHC or α -MHC/ α -MHC+ β -MHC) is significantly less than in age-matched normal tissue.

The expression of α -MHC, β -MHC, or both is preferably measured by quantitating mRNA levels, preferably by polymerase
20 chain reaction (PCR). MHC mRNA and protein levels are closely correlated (see Nadal-Grinard et al., *J. Clin. Invest.*, 84, 1693-1700 (1989)), and quantitative PCR is an extremely sensitive technique.

To perform the PCR assay, mRNA is obtained from a sample
25 of ventricular myocardial tissue and used to synthesize cDNA. Methods of extracting total cellular RNA from tissue and of preparing cDNA are well known.

Next, the cDNA coding for α -MHC, β -MHC, or both is amplified by PCR. PCR methods, equipment, and reagents are
30 well known and are available commercially.

The primers used in the PCR amplification should have sequences selected so that they hybridize to one of the

strands of the cDNA coding for α -MHC, β -MHC, or both. Methods of making nucleic acid molecules of a desired sequence are well known in the art. Of course, at least two primers must be used (one hybridizing to each of the strands of the cDNA), but more than one pair of primers can be used if it is desired to amplify more than one portion of the cDNA. The primers should be at least 18-20 bp in length with a G + C content greater than 40%. The specificity of the primers should be confirmed by Southern blotting.

Finally, the amplified PCR product is quantitated. This can be accomplished in a number of ways as is known in the art. For instance, the reaction mixture can be electrophoresed on agarose gels, and the amount of amplified PCR product of the expected size(s) can be determined. A labeled probe which hybridizes to an amplified PCR product can be used to allow for quantitation of the amplified PCR product. As another alternative, the primers can be labeled, or the nucleotides used during the PCR can be labeled, and the labels incorporated into the amplified PCR product can be quantitated.

As noted above, the probes or primers may be labeled to allow for quantitation of the cDNA coding for α -MHC, β -MHC, or both. Suitable labels and methods of attaching or incorporating them into nucleic acid molecules are well known.

25 Suitable labels include radioactive labels (e.g., ^{32}P), chemiluminescent labels, fluorescent labels (e.g., fluorescein, rhodamine), particulate labels (e.g., gold colloids), colorimetric labels (e.g., dyes), enzymes, and biotin.

30 As also noted above, labeled nucleotides can be used during PCR to generate an amplified PCR product which is labeled. The nucleotides are preferably labeled with

radioactive labels (e.g., ^{32}P) by methods well known in the art.

The quantities of the amplified PCR products are then related to the level of expression of α -MHC, β -MHC, or both in 5 the sample. Then, it is determined by statistical analysis if the expression of α -MHC, β -MHC, or both in the sample is significantly different than their expression in normal human ventricular myocardial tissue.

For instance, two particularly preferred PCR assays are 10 those described in Examples 1 and 3 below. In the assay described in Example 1, an internal standard cRNA transcribed from a synthetic gene is used to assure accurate quantitation of mRNA, and the levels of α -MHC and β -MHC mRNAs in sample and normal tissue are compared statistically. The assay described 15 in Example 3 only provides a ratio of α -MHC expression to total MHC expression (α -MHC/ α -MHC+ β -MHC), not absolute quantities of mRNA, and the ratios for sample and normal tissue are compared statistically. The assay described in Example 3 it is a relatively simple PCR assay to perform, and 20 it may also be used to quantitate other pairs of proteins besides α -MHC and β -MHC (see discussion below).

The mRNA coding for α -MHC, β -MHC, or both, can also be quantitated by contacting the mRNA, or cDNA prepared from it, with a nucleic acid probe having a sequence selected so that 25 the molecule hybridizes to mRNA or cDNA coding for α -MHC, β -MHC or both. To quantitate the mRNA, the probe is labeled. The probe should be as large as possible while retaining specificity. The probes may be made and labeled as described above for the primers. Stringent hybridization conditions 30 should be employed. In particular, it has been found that the 3' untranslated regions of the mRNAs coding for α -MHC and β -MHC have different lengths and substantially different

sequences (see Kurabayashi et al., *J. Clin. Invest.*, 82, 524-531 (1988)), and probes directed to these regions are preferred. The probes may also be used in an RNase protection assay, such as described in Bristow et al., *J. Clin. Invest.*, 5 92, 2737-2745 (1993).

The invention also provides a kit containing reagents useful for diagnosing myocardial failure. The kit comprises at least one container holding at least one nucleic acid molecule that hybridizes to DNA or RNA coding for α -MHC, β -MHC 10 or both. The nucleic acid molecule may be a PCR primer or a probe. The probes and primers may be labeled. The kit may comprise one or more additional containers holding additional probes or primers or an internal standard cRNA. The kit may contain other reagents and equipment useful in performing the 15 assay, including PCR reagents (e.g., polymerase, labeled or unlabeled nucleotides), reagents for extraction of mRNA, reagents for synthesizing cDNA from mRNA, buffers, salt solutions, containers, gels and membranes, etc.

Techniques other than those described above have been 20 used to quantitate α -MHC and β -MHC, and it may be possible to use these techniques in the method of the invention. However, as shown by the discussion in the Background section, these techniques may not have accurately determined the expression 25 of α -MHC and β -MHC in human myocardial tissue. Thus, the accuracy of these techniques must be determined, and the sensitivity and/or specificity of the techniques may need to be increased, before using them.

The invention also provides a method of treating myocardial failure in a human. The method comprises 30 administering an effective amount of an agent that directly causes an increase in the quantity of α -MHC in the myocardial tissue of the heart.

"Directly causing" is used herein to mean that the agent acts directly to increase the transcription of the α -MHC gene or to increase the translation of α -MHC mRNA by binding to the α -MHC gene, to an mRNA transcript of the gene, to a protein which binds to the gene or mRNA transcript, or to a compound which binds to the gene, mRNA transcript or a protein which binds to the gene or mRNA transcript. For instance, the agent may initiate or enhance transcription of the α -MHC gene by binding to an enhancer element of the α -MHC gene or to a transcription factor bound to an enhancer element of the gene. The agent could also stabilize the mRNA transcript, or bind to a compound that represses transcription, thereby blocking repression of transcription. Human α -MHC genomic and cDNA clones have been isolated, and some information is known about the regulation of α -MHC gene expression. See Kurabayashi et al., *J. Clin. Invest.*, 82, 524-531 (1988); Tsika et al., *Proc. Natl. Acad. Sci. USA*, 87, 379-383 (1990); Flink et al., *J. Biol. Chem.*, 265, 11233-1137 (1990); Chen et al., *Biochem. Biophys. Res. Commun.*, 188, 547-553 (1992); Morkin, *Circulation*, 87, 1451-1460 (1993). "Directly causing" is also used herein to mean that the agent acts directly to decrease the breakdown of the α -MHC protein by, e.g., binding to the α -MHC protein or to a compound that degrades or inactivates the α -MHC protein. Of course, a combination of agents, or a single agent, that performs two or more of the above functions (increases transcription, increases translation, decreases breakdown of protein) may be used.

As shown below, treatment of heart failure by blockade of β -adrenergic receptors results in increased expression of α -MHC and improved myocardial function. However, such treatments which increase the quantity of α -MHC indirectly as

a result of the improved myocardial function caused by the treatment are not included within the invention.

Agents that directly cause an increase in the quantity of α -MHC include thyroid hormones and analogs thereof. For instance, T₃ (3,5,3'-triiodo-L-thyronine) or T₄ (3,3',5,5'-tetraiodo-L-thyronine) may be used. One suitable thyroid hormone analog is 3,5-diiodothyropropionic acid, an analog known to increase the production of α -MHC in hypothyroid rats. Pennock et al., *J. Pharmacol. Exp. Therapeutics*, 263, 163-169 (1992). Pharmaceutical preparations of thyroid hormone are available commercially, and these commercially-available preparations may be used in the practice of the invention. See *Physicians Desk Reference*. Preferred is the synthetic thyroid hormone Synthroid.

Other suitable agents that directly cause an increase in the quantity of α -MHC can be identified using methods well known in the art. See, e.g., U.S. Patent No. 5,580,722 and Morkin, *Circulation*, 87, 1451-1460 (1993), the complete disclosures of which are incorporated herein by reference.

To treat a patient suffering from myocardial failure, an effective amount of an agent that directly causes an increase in the quantity of α -MHC in the myocardial tissue of the heart is administered to the patient. Effective dosage forms, modes of administration and dosage amounts, may be determined empirically, and making such determinations is within the skill of the art. It is understood by those skilled in the art that the dosage amount will vary with the activity of the particular agent employed, the severity of the myocardial failure, the route of administration, the rate of excretion, the duration of the treatment, the identity of any other drugs being administered to the patient, the age and size of the patient, and like factors well known in the medical art. In

general, a suitable daily dose of an agent of the present invention will be that amount of the agent which is the lowest dose effective to produce a therapeutic effect. A suggested daily dosage of thyroid hormone for treatment of myocardial failure is about 50-200 µg/day of synthetic thyroid hormone (e.g., Synthroid). However, the total daily dosage of the agent will be determined by an attending physician within the scope of sound medical judgment. If desired, the effective daily dose may be administered as two, three, four, five, six or more sub-doses, administered separately at appropriate intervals throughout the day.

The agents of the present invention may be administered to a patient for therapy by any suitable route of administration, including orally, parenterally, and topically. The preferred route of administration is orally.

While it is possible for an agent of the present invention to be administered alone, it is preferable to administer the compound as a pharmaceutical formulation (composition). The pharmaceutical compositions of the invention comprise one or more of the agents of the invention as an active ingredient in admixture with one or more pharmaceutically-acceptable carriers and, optionally, with one or more other compounds, drugs or other materials. Each carrier must be "acceptable" in the sense of being compatible with the other ingredients of the formulation and not injurious to the patient. Suitable carriers are well known in the art.

Pharmaceutical formulations of the present invention include those suitable for oral, topical and/or parenteral administration. Regardless of the route of administration selected, the agents of the present invention, are formulated

into pharmaceutically-acceptable dosage forms by conventional methods known to those of skill in the art.

The amount of active ingredient which will be combined with a carrier material to produce a single dosage form will vary depending upon the host being treated, the particular mode of administration and all of the other factors described above. The amount of active ingredient which will be combined with a carrier material to produce a single dosage form will generally be that amount of the agent which is the lowest dose effective to produce a therapeutic effect.

Methods of preparing pharmaceutical formulations or compositions include the step of bringing into association an agent of the present invention with the carrier and, optionally, one or more accessory ingredients. In general, the formulations are prepared by uniformly and intimately bringing into association an agent of the present invention with liquid carriers, or finely divided solid carriers, or both, and then, if necessary, shaping the product. Suitable and necessary accessory ingredients are well known in the art.

Another embodiment of the present invention is the treatment of myocardial failure by gene therapy to increase α -MHC expression. The gene therapy method of the invention comprises delivering a transgene coding for α -MHC to a human so that the α -MHC is expressed in at least the myocardial tissue of the heart of the recipient. Thus, "agents that directly cause an increase in the quantity of α -MHC in the myocardial tissue of the heart" include transgenes coding for α -MHC.

The α -MHC transgene is constructed and cloned by standard methods known in the art. Such standard methods are disclosed, for example, in Sambrook et al., 1989, *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Labs Press.

The reference Sambrook et al., *ibid.*, is incorporated herein by reference in its entirety.

As noted above, genomic and cDNA clones coding for human α-MHC are known. Other clones can be isolated using standard methods known in the art. Alternatively, DNA coding for human α-MHC can be synthesized by chemical synthetic methods known in the art using the known sequence. The transgene may contain a single copy or multiple copies of the α-MHC coding sequence (increasing the copy number of the coding sequence will increase expression of α-MHC).

In addition to the DNA coding for α-MHC, the transgene is constructed to include a promoter selected to drive expression of the α-MHC exclusively in the heart and in at least the myocardial tissue. Preferably, the promoter is an α-MHC promoter.

In addition to the promoter, the transgene will contain other expression control sequences necessary or desirable for proper expression and processing of the α-MHC. These expression control sequences and the promoter will be operatively linked to the α-MHC-encoding DNA. The phrase "operatively linked" refers to linking of nucleic acid sequences in the transgene in a manner such that the α-MHC can be expressed in cardiac cells when the transgene is integrated into a host genome. The additional expression control sequences are well known in the art and include sequences which control the initiation, elongation, and termination of transcription (such as enhancer sequences and polyadenylation sequences).

Methods and materials for gene therapy are well known in the art. See Culver, *Gene Therapy: A Primer for Physicians* (Revised 2nd ed., 1996), U.S. Patents Nos. 5,521,291, 5,460,831 and 5,559,099, PCT application WO 96/14876, all of

which are incorporated herein by reference in their entirety. See also, Kirshenbaum, et al., *J. Clin. Invest.*, **92**, 381-387 (1993) and Drazner et al., *J. Clin. Invest.*, **99**, 288-296 (1997). In particular, suitable methods and vehicles for delivery of transgenes are known and may be used to deliver the α -MHC transgene. For instance, naked DNA can be injected directly into the myocardium. Also, targeted vehicles may be used for delivery of the transgene to myocytes. A targeted vehicle is one which includes a component that allows the vehicle to bind to, and deliver the transgene to, a specific cell or tissue. Such targeted vehicles are known in the art. The transgene may be included in a viral vector for delivery to myocytes. Such viral vectors are known in the art. Preferably, the transgene is incorporated into an adenoviral vector for delivery. See Kitsis, et al., *Proc. Natl. Acad. Sci. USA*, **88**, 4138-4142 (1991); Kass-Eisler et al., *Proc. Natl. Acad. Sci. USA*, **90**, 11498-11502 (1993); Akhter, et al., *Proc. Natl. Acad. Sci USA*, **94**, 12100-12105 (1997). The transgene is preferably infused directly into the heart by injecting it into the coronary artery, thereby ensuring the greatest amount of transgene absorption into myocytes. The transgene preferably integrates into the host cell's genome to provide for stable transformation.

Finally, the invention provides a method of quantitating the expression of a first protein relative to the expression of a second protein or to the total expression of the first and second proteins. The method comprises obtaining a sample of cells or tissue expressing the first protein and the second protein, extracting RNA from the cells or tissue, preparing cDNA from the RNA, amplifying the cDNA coding for the first and second proteins by PCR using primers that hybridize to cDNA coding for the first protein, the second protein or both,

and quantitating the amplified PCR products. All of these techniques are described above.

The quantities of the amplified PCR products are then related to the levels of protein expression in the sample, and
5 the expression of a first protein relative to the expression of a second protein or to the total expression of the first and second proteins is calculated. Then, it is determined by standard statistical analysis if the calculated relative expression of the first protein in the sample cells or tissue
10 is significantly different than it is in normal cells or tissue.

Any pair of proteins can be quantitated in this manner, but the pair preferably have sequences similar enough so that the cDNAs coding for them can be amplified by the same pair of
15 primers (so that any errors due to differences in primer efficiencies are readily avoided), but different enough so that the PCR products can be differentiated in some manner (e.g., size, different enzyme cleavage sites). Examples of such proteins include α -MHC and β -MHC (see Example 3), mutated
20 and unmutated versions of the same protein (e.g., gene therapy), the same protein from different species (e.g., transgenic animals), proteins produced as a result of alternate splicing. For less similar proteins, differences in PCR conditions and primer efficiencies must be accounted for.
25 For instance, one of the proteins of the protein pair could be one whose expression is not expected to change under the conditions being studied. For example, sarcoplasmic reticulum Ca^{2+} ATPase (SRCA) expression does not change significantly in failing hearts as compared to normal hearts (see Examples 1-3
30 and 5), and changes in the ratio of α -MHC expression to SRCA expression should accurately reflect changes in α -MHC expression.

EXAMPLES

EXAMPLE 1: Measurement of expression of MHC isoforms in endomyocardial tissue by PCR

5 The amount of mRNA coding for α -MHC, β -MHC, β_1 -adrenergic receptor (β_1 AR), β_2 -adrenergic receptor (β_2 AR), sacroplasmic reticulum Ca^{2+} ATPase (SRCA), and atrial natriuretic factor or peptide (ANP) in total mRNA extracted from endomyocardial biopsy specimens was measured by quantitative PCR. The 10 endomyocardial specimens were taken from the hearts of 29 subjects with biventricular failure from idiopathic dilated cardiomyopathy (IDC), 7 subjects with right ventricular failure from primary pulmonary hypertension (PPH), and 8 nonfailing (NF) controls.

15 The IDC and PPH donors were all ambulatory subjects who were not end-stage from the standpoint of clinical condition or intravenous inotropic support. Five of the PPH patients were New York Heart Association (NYHA) class III, one was class II, and one was class IV. Twenty-one of the IDC 20 patients were NYHA class III, five were class II, and three were class IV.

The NF control samples were obtained from: five cancer patients about to begin chemotherapy with IL-4 or liposomal adriamycin; two subjects with normal systolic function and 25 atypical chest pain or unexplained dyspnea on exertion who were biopsied to rule out myocarditis or cardiomyopathy; and a subject with normal right ventricle function and minimal left ventricle dysfunction who was biopsied to rule out an infiltrative myocardial process 72 hours after being 30 resuscitated from a cardiac arrest that was ultimately attributed to coronary artery spasm. Six of the NF controls were NYHA class I, one was NYHA class II, and the subject who

had arrested returned to class I several weeks after being evaluated.

Hemodynamics for these tissue donors are given in Table 1 below. RVEF and LVEF are right ventricle ejection fraction 5 and left ventricle ejection fraction, respectively, RA is right atrial mean pressure, PAP is pulmonary artery mean pressure, and PW is pulmonary wedge mean pressure.

Total RNA was extracted from 2-4 endomyocardial biopsies (a total of 4-8 mg) taken from distal right ventricle (RV) 10 septum. The biopsies were performed with a Mansfield 2.2 mm jaw size (Boston Scientific Corp., Watertown, MA) bioptome under echocardiographic guidance to ensure proper positioning of the biopsy forceps. Total RNA was extracted by the guanidinium thiocyanate phenol-chloroform method using RNA 15 STAT-60, as previously described. Feldman et al., *Circulation*, 83, 1866-1872 (1991); Bristow et al., *J. Clin. Invest.*, 92, 2737-2745 (1993). A double extraction was used to eliminate small amounts of DNA contamination.

The mRNA was quantitated by PCR, as previously described 20 in Feldman et al., *Circulation*, 83, 1866-1872 (1991) and Bristow et al., *J. Clin. Invest.*, 92, 2737-2745 (1993), the complete disclosures of which are incorporated herein by reference, with some modifications described below.

Briefly, this PCR technique (referred to herein as "RT-QPCR") is based on the simultaneous reverse transcription of 25 an internal standard cRNA and an mRNA of interest, followed by simultaneous PCR amplification with the same primers of the cDNAs produced from the internal standard cRNA and mRNA of interest. The internal standard cRNA is designed to produce 30 a cDNA of smaller size than the PCR product generated from the transcript of interest.

The internal standard cRNA is prepared by constructing a synthetic gene which contains a coding region comprising a DNA sequence complementary to the primers used to specifically amplify a region of the cDNA prepared from the mRNA that is to
5 be measured. The synthetic gene is driven by the T7 RNA polymerase promoter, and *in vitro* transcription of the synthetic gene produces the internal standard cRNA. The synthetic gene also contains a polyadenine tract at its 3' end to facilitate reverse transcription. The internal standard
10 cRNA is used as a template for reverse transcriptase in the same reaction that generates cDNA from the mRNA of interest, and a cDNA corresponding to the internal standard cRNA is produced along with the cDNA corresponding to the mRNA of interest.

15 One internal standard cRNA can be used to measure multiple (4-20) mRNAs by constructing a synthetic gene having a coding region comprising DNA sequences complementary to primers used to specifically amplify a region of each of the multiple cDNAs prepared from the mRNAs in the sample that are
20 to be measured. Such a synthetic gene was used in the assays described in the present example. It contained sequences complementary to the mRNAs coding for α -MHC, β -MHC, β_1 AR, β_2 AR, SRCA and ANP. In particular, the gene's coding region contained sequences corresponding to that of the primers used
25 to amplify the α -MHC, β -MHC, β_1 AR, β_2 AR, SRCA and ANP cDNAs. Thus, the cDNA generated from the internal standard cRNA could be amplified by the same primers used to amplify the cDNAs generated from the mRNAs of interest. However, the PCR products produced from the internal standard cDNA were smaller
30 than the PCR products produced from the cDNAs generated from the mRNAs of interest.

When possible, the sequences of the primers were selected to cross splice junctions, so that genomic DNA would not be amplified. For intronless genes, such as those of the adrenergic receptors, the extreme sensitivity (into the 5 zeptomole range) of the assay can lead to genomic DNA amplification. An RT(-) control (no reverse transcription) was included to detect this possibility.

Further, in the assay for β_1 AR mRNA, the reverse transcription (RT) and PCR conditions were modified to 10 increase the RT and amplification efficiencies, to eliminate significant genomic DNA contamination, and to obtain robust cDNA amplification in 30 PCR cycles or less. These modifications included double extraction of RNA with RNA STAT 60TM (Tel-Test, Friendswood, TX) and multiple changes in the 15 RT assay conditions, including adding 2% DMSO, using oligo d(T₁₅) primers to gain specificity for mRNA, increasing the concentration of Moloney murine leukemia virus (MMLV) reverse transcriptase by 50%, and carrying out the RT reaction in a thermocycler. The thermocycler protocol included an initial 20 15 minutes at 37°C, ramping the temperature up to 45°C over 15 minutes and holding it there for an additional 15 minutes, and finally inactivating the transcriptase at 95°C for 10 minutes. With these modifications, a 202 bp region of the β_1 AR cDNA 25 reverse transcribed from mRNA in total RNA as the starting material was amplified in 30 cycles or less of PCR without genomic amplification. These modifications allowed for measurement of β_1 AR mRNA abundance in total RNA extracted from small (1-3 mg) samples of human ventricular myocardium.

As determined using *in vitro* transcribed mRNA relative to 30 the internal standard, the efficiency of reverse transcription was 7.5 times greater for β_2 AR mRNA compared with β_1 AR mRNA, and the mRNA abundance of β_1 AR mRNA determined by ribonuclease

protection was 6-10 times that determined by quantitative RT-PCR. Therefore, in the RT-PCR determinations in this study, the calculated amount of β_1 AR mRNA was multiplied by 7.5.

Three reverse transcriptase reactions were necessary to ensure collinear amplification of the internal standard and cDNAs of interest. Stated another way, it is necessary for the reverse transcribed internal standard and the unknown mRNA to be within 10-fold of one another before amplification in order to obtain collinearity. In general, one reverse transcriptase reaction each was used for low (β ARs), medium (ANP, α -MHC), and high (SRCA, β -MHC) abundance messages. The precision of the assay as assessed by repeat measurements on the same sample yielded a coefficient of variation of 12.8% for β_1 AR mRNA, and 10-20% for all gene products tested ($n = 8$). All PCR products were confirmed to be the expected cDNA by subcloning the PCR product using the PGEM-T vector system (Promega, Madison, WI), and then sequencing the cloned fragment.

The sequences of the MHC primers used in the RT-QPCR assay are presented below. The nucleotide numbering referred to is that used in Kurabayashi et al., *J. Clin. Invest.*, 82, 524-531 (1988).

The 5' primer for α -MHC corresponds to nucleotides 1327-1347 of Kurabayashi et al., *J. Clin. Invest.*, 82, 524-531 (1988). Its sequence is:

5'-ATCAAGGAGCTCACCTACCAG-3' SEQ ID NO:1.

The 3' primer for α -MHC corresponds to nucleotides 1592-1572. Its sequence is:

3'-CACTCCTCATCGTGCATTTTC-5' SEQ ID NO:2.

SEQ ID NO:2 anneals to a portion of the α -MHC cDNA, including the end of the coding sequence, which differs substantially from the sequence of the corresponding portion of the β -MHC

cDNA. See Kurabayashi et al., *J. Clin. Invest.*, 82, 524-531 (1988).

The sequence of the 5' primer for β -MHC is the same as that of the 5' primer for α -MHC:

5 5'-ATCAAGGAGCTCACCTACCAG-3' SEQ ID NO:1.

The 3' primer for β -MHC corresponds to nucleotides 1662-1643. Its sequence is:

3'-AGCTGTTACACAGGCTCCAG-5' SEQ ID NO:3.

SEQ ID NO:3 anneals to a portion of the 3' noncoding region of 10 the β -MHC cDNA which differs substantially from the sequence of the corresponding portion of the α -MHC cDNA. See Kurabayashi et al., *J. Clin. Invest.*, 82, 524-531 (1988).

As noted above, the coding region of the synthetic gene contained sequences corresponding to that of the primers used 15 to amplify the α -MHC and β -MHC cDNAs. Thus, the sequence of the α -MHC portion of the synthetic gene was:

5'-ATCAAGGAGCTCACCTACCAGGTGAGGAGTAGCACGTAAAAG-3'
3'-TAGGCCCGCAGTGGATGGTCCACTCCTCATCGTGCATTTTC-5'

20 SEQ ID NOS:4 and 5.

The sequence of the β -MHC portion of the synthetic gene was:

25 5'-ATCAAGGAGCTCACCTACCAGTCGACAATGTGTCCGAGGTC-3'
3'-TAGGCCCGCAGTGGATGGTCAGCTGTTACACAGGCTCCAG-5'

SEQ ID NOS:6 and 7.

All nucleotides were synthesized by Operon Technologies, 30 Inc. For the sequences of the other primers, see Lowes et al., *J. Clin. Invest.*, 100, 2315-2324 (1997), the complete disclosure of which is incorporated herein by reference.

First-strand cDNA was synthesized from the total RNA using reverse transcriptase (SuperScript II RT, BRL Life

Technologies, Inc., Gaithersburg, MD) and oligo dT (BRL Life Technologies Inc.) from 1 µg of extracted total RNA according to manufacturer's instructions.

The PCR was carried out in a total volume of 100 µl
5 containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.001%
(w/v) gelatin, 160 µM dNTPs, 13 pmol of each primer, and 1.25
units of *Thermus aquaticus* DNA polymerase (*Taq* polymerase)
(Perkin-Elmer/Cetus Corp., Norwalk, Conn.). A trace amount of
32P-labeled 3' primer was added to provide 3 x 10⁶ cpm. The
10 mixture was amplified with a thermal cycling system
(TempCycle, Coy Corp., Ann Arbor, Mich.). Each reaction also
contained a known quantity of internal standard cDNA prepared
by reverse transcription of the synthetic gene. Amplification
temperatures were 94°C for 1.5 minutes for denaturation, 50-
15 55°C for 1.5 minutes for primer annealing, and 72°C for 1.5
minutes for primer extension. The primer-annealing
temperature varied according to the specific primers being
used for amplification.

The amount of DNA synthesized was quantified by measuring
20 the amount of ³²P-end labeled primer incorporated into the PCR
products. To do so, 10 µl of each PCR reaction mixture was
removed and electrophoresed in an agarose gel. Gels were
visualized with ethidium bromide staining and indirect UV
irradiation. Bands representing amplification products from
25 the cDNA of interest and the internal standard cDNA were cut
out of the gel. Radioactivity in the bands was determined by
Cerenkov counting. To ensure that measurements were performed
during the exponential phase of amplification, the PCR
reaction mixtures were sampled during cycles 15-33 of
30 amplification.

Amplification curves for the internal standard cDNA and
the cDNA generated from the mRNA of interest were constructed

by plotting the amount of radioactivity in the excised gel bands against the number of PCR cycles. Since a known amount of internal standard cRNA (determined spectrophotometrically) was carried through the cDNA synthesis and amplification, the
5 amount of an mRNA of interest could be determined by extrapolation from the internal standard cDNA standard curve. It is essential that this extrapolation be performed during the exponential phase of amplification and from regions of the amplification curves that are collinear.

10 Bivariate statistical analyses were performed with Statview 512™ (Brainpower, Inc., Calabasas, CA) and multivariate and nonparametric analyses were performed with SAS (SAS Institute, Cary, NC). Unless otherwise specified, data are expressed as mean \pm standard error. The two-sided
15 significance level was $P < 0.05$. Three-group comparisons were performed with ANOVA for continuous variables and by contingency table analysis for binary variables. With a significant three-group comparison, specific two-group comparisons were performed with the Scheffe or Bonferroni
20 multiple comparisons procedures. When data were not normally distributed as assessed by the Shapiro-Wilk test, the ANOVA and t test results were confirmed by the Kruskal-Wallis and Wilcoxon tests, respectively. Univariate and multivariate relationships between continuous variables were assessed with
25 stepwise linear regression, using $P = 0.15$ to enter and to remove.

The results are presented in Table 2. As can be seen, compared with the nonfailing groups, β_1 AR mRNA abundance is reduced in both the PPH and IDC groups (by ~ 50% in the IDC
30 group and 60% in the PPH group). Surprisingly, the PPH group exhibited an increase in β_2 AR mRNA abundance of 85% compared with the nonfailing group and of 152% compared with the IDC

group. The data for ANP are consistent with an increase in ANP gene expression in both the PPH and IDC groups. There were no differences in expression of SRCA mRNA among the three groups.

5 The RV endomyocardium of the nonfailing hearts contained a substantial amount of the α isoform of MHC ($\sim 37.0 \times 10^5$ molecules/ μ g total RNA, range 10.7-72.2). However, β -MHC is the dominant isoform in all three groups. Both PPH and IDC exhibited a marked decrease in α -MHC mRNA abundance (by 75% in
10 PPH and 60% in IDC). As can be seen, compared with the nonfailing group there is a tendency for the abundance of β -MHC to be slightly higher in both PPH and IDC groups ($P = 0.06$ by ANOVA). The percentage of total MHC represented by the α -MHC isoform decreased from 23.1 to 5.6% in PPH and to 7.6% in
15 IDC (both $P < 0.001$). The change in the percentage of β -MHC varied from 76.9% in nonfailing ventricles to 94.4% in PPH and 92.2% in IDC. The MHC data are surprising because an MHC isoform shift was not thought to occur in failing human hearts (see Background section).

20 The data in Table 2 confirm previous findings of a down-regulation of β_1 AR mRNA in the failing human left ventricle (LV) (Bristow et al., *J. Clin. Invest.*, **92**, 2737-2745 (1993)) and in endomyocardial biopsies taken from failing RVs (Englehardt, et al., *J. Am. Coll. Cardiol.*, **27**, 146-154
25 (1996)). The data also confirm previous reports of an up-regulation of ANP (LV free wall as starting material) (Feldman et al., *Circulation*, **83**, 1866-1872 (1991)). The results do not confirm previous data from end-stage IDC explanted hearts showing that SRCA mRNA is down-regulated (Mercadier et al., *J.
30 Clin. Invest.*, **85**, 305-309 (1990)), but are in agreement with some investigators' measurements of SRCA protein mass (Movsesian et al., *Circulation*, **90**, 653-657 (1994)) and

activity (Movsesian et al., *Circ. Res.*, **65**, 1141-1144 (1994)) in end-stage, explanted human hearts.

Finally, recent experiments (data not shown) demonstrate that the amounts of α -MHC and β -MHC mRNA and changes in the 5 mRNA levels correlate with the amounts and changes in α -MHC and β -MHC protein measured in the same tissue samples. These results confirm the results of others who have also found that MHC mRNA and protein levels are closely correlated (Nadal-Ginard et al., *J. Clin. Invest.*, **84**, 1693-1700 (1989)).

10

Table 1

Group	RVEF (%)	LVEF (%)	RA (mmHg)	PAP (mmHg)	PW (mmHg)	Cardiac Index (L/min/m ²)
NF(n=8)	52±2	59±3	2.5±1.9	14.8±3.5	5.0±1.9	4.46±0.46
PPH(n=7)	*29±2	53±3	9.1±2.3	*49.6±4.8	6.1±1.2	*2.06±0.18
IDC(n=29)	33±3	*23±1	6.7±1.1	*27.9±1.9	*15.0±1.6	*2.49±0.14

*p<.05 vs. NF by ANOVA/Scheffe Test

#p<.05 vs. IDC by ANOVA/Scheffe Test

@p<.05 vs.PPH by ANOVA/Scheffe Test

Table 2

Source of RNA (number)@	mRNA abundance, molecules $\times 10^5/\mu\text{g}$ total RNA (\pm SEM)						MHC isoform %	
	$\beta_1\text{AR}$	$\beta_2\text{AR}$	ANP	SRCA	$\alpha\text{-MHC}$	$\beta\text{-MHC}$	% $\alpha\text{-MHC}$	% $\beta\text{-MHC}$
IDC(n=29)	*1.6 \pm 0.4	1.9 \pm 0.1	*108 \pm 20	76.0 \pm 5.0	*14.8 \pm 1.8	183 \pm 14	*7.6 \pm 1.1	*92.2 \pm 1.0
PPH(n=7)	*1.2 \pm 0.1	**4.8 \pm 1.1	*140 \pm 45	75.0 \pm 4.6	*9.2 \pm 1.8	183 \pm 28	*5.6 \pm 2.0	*94.4 \pm 2.0
NF(n=8)	3.1 \pm 0.8	2.7 \pm 0.6	35.0 \pm 12.9	80.3 \pm 13.0	37.0 \pm 6.5	108 \pm 15	23.1 \pm 0.8	76.9 \pm 0.8

* p<.05 vs. NF (ANOVA)

p<.05 vs. IDC (ANOVA)

@ The average age, in years, of the tissue donors was:

IDC 55.0 \pm 2.0 (p<0.05 vs PPH by ANOVA);
 PPH 36.6 \pm 2.8 (p<0.05 vs. IDC by ANOVA); and
 NF 49.1 \pm 4.6.

EXAMPLE 2: Measurement of MHC isoforms in three different regions of human ventricular myocardium

5 The amount of mRNA coding for $\alpha\text{-MHC}$, $\beta\text{-MHC}$, $\beta_1\text{AR}$, $\beta_2\text{AR}$, SRCA and ANP in total mRNA extracted from different areas of human hearts was measured as described in Example 1. The specimens were taken from the RV endomyocardium ("RV endo"), from full thickness mid RV free wall (RV FW), and from full 10 thickness mid LV free wall (LV FW). The RV endo samples were taken by simulated endomyocardial biopsies. The hearts were taken from 6 patients with end-stage biventricular failure (LVEF = 14 \pm 2%) from idiopathic dilated cardiomyopathy (IDC) prior to transplantation (age 45 \pm 5 years) and from 6 15 prospective organ donors (NF) (age 38 \pm 7 years). The hearts of the prospective organ donors were ultimately rejected for transplantation for non-cardiac reasons, but they had no history of cardiac disease and had normal cardiac function by

medical history and echocardiograph or inspection at the time of organ harvest (LVEF \geq 45%).

The results are presented in Table 3 below (mRNA molecules $\times 10^5/\mu\text{g}$ total RNA \pm SEM). As can be observed, α -MHC expression is significantly decreased in all three types of tissue from IDC hearts, as compared to NF hearts. The β -MHC expression is increased in all three types of tissue and is significantly increased in RVFW and LVFW, as compared to NF hearts. Also, there is good agreement between absolute levels of mRNA coding for α -MHC, β -MHC, $\beta_1\text{AR}$, $\beta_2\text{AR}$, and ANP in the three sampled regions. Levels of SRCA mRNA appear to be slightly lower in the LVFW as compared to either RV location. Note also that the data are in agreement with the changes in mRNA levels detected in endomyocardial biopsy tissue from *in vivo* sampled hearts (see Table 2 above).

Similar changes in expression of α -MHC and β -MHC in IDC are observed in all three regions. Gene expression in RV endomyocardium is, therefore, representative of gene expression in the more functionally relevant RV free wall and can be used to assess RV function generally. Further, these results also show that gene expression in RV endomyocardium can be used to assess LV function, when LV and RV function are equally affected, as in the IDC subjects.

Table 3

Region	β_1 -AR			β_2 -AR			ANP			SRCA			α -MHC			β -MHC		
	NF	IDC	NF	IDC	NF	IDC	NF	IDC	NF	IDC	NF	IDC	NF	IDC	NF	IDC		
RV endo	4.9 \pm .4	*3.1 \pm .3	2.5 \pm .3	2.0 \pm .3	34 \pm 2	*121 \pm 26	86 \pm 5	75 \pm 9	46 \pm 7	*9.4 \pm 1.1	111 \pm 7	142 \pm 5						
RV FW	4.7 \pm .4	*3.3 \pm .4	2.1 \pm .5	1.8 \pm .3	32 \pm 2	*92 \pm 18	101 \pm 12	79 \pm 10	54 \pm 6	*9.7 \pm 1.6	104 \pm 11	*163 \pm 6						
LV FW	4.8 \pm .6	*2.9 \pm .6	2.1 \pm .4	1.6 \pm .2	38 \pm 5	*110 \pm 24	668 \pm 9	656 \pm 6	46 \pm 9	*8.9 \pm 1.9	118 \pm 10	*146 \pm 18						

*p<.05 vs. NF

#p<.10 vs. NF

δp<.10 vs. RV endo or RV FW (ANOVA)

EXAMPLE 3: Measurement of MHC isoforms in human left ventricles

The amount of mRNA coding for α -MHC and β -MHC in total
5 mRNA extracted from left ventricles (LVs) was measured. The hearts were taken from 14 prospective organ donors (age 34.4 \pm 13.9 years) and from 19 patients undergoing cardiac transplantation for chronic end-stage heart failure (age 50.1 \pm 14.6 years).

10 The 14 prospective organ donors (control subjects) were motor vehicle accident victims, cerebral vascular accidents, and suicides by gunshot. They had no cardiac history and no significant structural abnormalities on cardiac donor screening using echocardiography and, in subjects > 50 yr of
15 age, coronary angiography. All control subjects were maintained under intensive care, but seven of the hearts (NF) were excluded ultimately from heart donation because of age, body size, or blood type incompatibility. In all NF heart cases, the echocardiogram revealed normal LV systolic
20 function. The remaining 7 of the 14 control subjects were identified as having acute donor heart dysfunction (DHD), which is thought to be due to myocardial injury from massively increased sympathetic output accompanying brain injury. These hearts were excluded from heart donation because of decreased
25 LV systolic function (ejection fraction \leq 45%). In all DHD cases, the echocardiogram revealed diffuse hypokinesis without segmental LV wall motion abnormalities.

The 19 heart failure patients had the following diagnoses: idiopathic dilated cardiomyopathy (IDC) (6),
30 valvular cardiomyopathy (VHD) (2), end-stage hypertrophic cardiomyopathy (1), orthotopic heart transplantation (1), or ischemic cardiomyopathy (IHD) with history of coronary artery disease and at least one myocardial infarction(9).

The hearts were placed in ice-cold oxygenated saline immediately after removal, and myocardial specimens were gathered within 10 minutes after explantation. A transmural specimen was taken from the medial part of the left ventricular free wall when possible, and midway between the apex and base, at least 2 cm away from the scar in patients with myocardial infarction. Immediately after sampling, myocardial specimens were frozen in liquid nitrogen and stored at -80°C until used.

10 Total RNA was isolated from 35-276 mg of frozen left ventricular myocardium specimen using a modification of the acid guanidinium thiocyanate-phenol-chloroform method. Chomczynski and Sacchi, *Anal. Biochem.*, 162, 156-159 (1987). Briefly, the frozen tissue was homogenized at 4°C in 2.0 ml of

15 RNA STAT-60 (Tel-Test "B", TelTest, Inc., Friendswood, TX) with an Ultra-Turrax (13,500 rpm for 30 seconds x 2, IKA-Works, Cincinnati, OH). After centrifugation (12,000 g for 15 minutes), the aqueous phase was collected, and RNA was precipitated by the addition of the same volume of

20 isopropanol. Then, the RNA pellet was washed with 70% ethanol and resuspended in water treated with diethyl pyrocarbonate to inactivate ribonucleases. The concentration of extracted total RNA was determined using a spectrophotometer (Hitachi U-2000, Hitachi Sci. Instrs., Mountain View, CA).

25 First-strand cDNA was synthesized from the total RNA using reverse transcriptase (SuperScript II RT, GIBCO BRL, Gaithersburg, MD) and random hexamer (GIBCO BRL) from 2 µg of extracted total RNA according to manufacturer's instructions.

Two primers were designed from reported α -MHC and β -MHC
30 cDNA sequences and were synthesized by GIBCO BRL. The sequences of these primers were:

5' primer - 5'-AGCAGAAGCGCAACGCAGAGT-3' SEQ ID NO:8
3' primer - 3'-GTTCAAGGCCTTCCACGTCGT-5' SEQ ID NO:9.

These primers are identical in sequence between α -MHC and β -MHC, and the amplified 217-bp sequence was 96% identical between α -MHC and β -MHC. The α -MHC and β -MHC cDNAs were, therefore, amplified with equal efficiency. The 5' primer is located in exon 36 and 37 of the α -MHC and β -MHC genes, respectively. The 3' primer is located in exon 38 and 39 of the α -MHC and β -MHC genes, respectively. The two exon/intron boundaries are conserved. The amplified regions correspond to nucleotides 5492-5708 and 5572-5788 in α -MHC and β -MyHC cDNA, respectively. Matsuoka et al., *Am. J. Med. Genet.*, **41**, 537-547 (1991); Jaenicke et al., *Genomics*, **8**, 194-206 (1990).

The PCR was carried out in a total volume of 50 μ l containing 10 mM Tris-HCl, 50 mM KCl, 1.5 mM MgCl₂, 0.2 mM dNTPs, 15 pmole of each primer, and 2.5 units of *Thermus aquaricus* DNA polymerase (*Taq* polymerase) (Perkin-Elmer/Cetus Corp, Norwalk, CT). The mixture was amplified with a thermal cycling system (OmniGene Hybaid Ltd., Middlesex, UK) with the following conditions. After the "hot start" at 94°C for 3 minutes, amplification was done by 21 cycles of the following program: 94°C for 45 seconds for denaturation, 55°C for 45 seconds for annealing, and 72°C for 90 seconds for primer extension. Then, the mixture was kept at 72°C for 10 minutes and held at 30°C.

The amplified 217-bp fragment was digested with endonucleases, PstI or SacI (New England BioLabs, Inc., Beverly, MA). Only the β -MHC product has PstI sites, while both MHC products have SacI sites. Therefore, the 217-bp bands remaining after digestion with PstI or SacI correspond to the amplified α -MHC cDNA and the background, respectively.

When SacI digestion did not completely eliminate the background (a very rare occurrence), the assay was not considered valid and was re-run. Digestion was performed in a total volume of 20 μ l containing 5 μ l of PCR product, with appropriate buffers, with (PstI, 20 units, or SacI, 20 units) or without (replaced by distilled water) restriction enzymes, at 37°C for 3 hours according to the manufacturer's instructions.

The digested fragments were subjected to 8% polyacrylamide gel electrophoresis (8 μ l of 20 μ l reaction, room temperature, 10V/cm constant) with a Mighty Small II (Hoefer Pharmacia Biotech, Inc., San Francisco, CA). The gel was stained with CYBR Green I (diluted 1:10,000, Molecular Probes, Inc., Eugene, OR) for 60 minutes with gentle agitation. CYBR Green I is an intercalating fluorescent dye that binds specifically to nucleic acids. The digested fragments were clearly separated from the original 217-bp band. The fluorescence signal of the 217-bp bands was detected using a fluorescence imaging system (STORM, Molecular Dynamics, Sunnyvale, CA) and the intensity was evaluated with a quantification program (ImageQuant, Molecular Dynamics, CA). The relative amount of amplified α -MHC cDNA to total MHC (α -MHC + β -MHC) was expressed as follows:

Amplified α -MHC cDNA ratio (%) =
$$100 \frac{[SI_{PstI} - SI_{SacI}]}{[SI_{Enz-} - SI_{SacI}]}$$
,

where SI_{PstI} , SI_{SacI} and SI_{Enz-} are the signal intensity (in arbitrary units) of the 217-bp band after incubation with PstI, with SacI, or without endonucleases, respectively.

The results are presented in Table 4 below. Note that the NF group had a measurable amount of the fast contracting α -MHC isoform, consistent with the measurements made in RV

endomyocardial biopsy material (Example 1). The DHD group also had a measurable amount of α -MHC. Failing human LVs exhibited a significant reduction in α -MHC as compared to NF controls. MHC mRNA and protein levels are closely correlated
5 (see Nadal-Grinard et al., *J. Clin. Invest.*, **84**, 1693-1700 (1989) and Example 1), and these differences in gene expression should be reflected in the amount of α -MHC protein. Assuming a 4-fold difference in speed of contraction in favor
10 of the α -isoform, these changes would result in a 44-46% reduction in velocity of shortening in the failing group. The mean values of total MHC cDNA were not statistically different among all three patient groups: NF - 827 ± 215 , DHD - 1062 ± 570 , and F - 951 ± 449 (arbitrary units).

15

Table 4

Group	LVEF (% \pm SEM)	α -MHC mRNA (% \pm SEM)
NF (n=7)	63 \pm 5	33.3 \pm 18.9
DHD (n=7)	\$29 \pm 11	@35.4 \pm 16.5
F (n=19)	\$18 \pm 6	*2.2 \pm 3.5

25 NF=nonfailing
DHD=donor heart dysfunction
F=all heart failure patients
* p<.05 vs. NF and DHD (ANOVA)
@p<0.05 us F (ANOVA)
\$p<0.05 us NF (ANOVA)

30

The data are expressed as mean \pm standard deviation. The data were analyzed by the nonparametric analogues of ANOVA and the two-tailed *t* test, which are the Kruskal-Wallis and the Wilcoxon rank-sum test, respectively. By Kruskal-Wallis,
35 P = 0.0001. By Wilcoxon, for comparisons of NF vs. DHD, NF

vs. F, and DHD vs. F hearts, *P* values were 1.000, 0.0001, and 0.0001, respectively.

The accuracy of the RT-PCR was verified by performing a ribonuclease protection assay (RPA) using the same specimens.

5 The RPA was performed as described in Nakao et al., *J. Clin. Invest.*, 100, 2362-2370 (1997), the complete disclosure of which is incorporated herein by reference. The α -MHC ratios obtained by RPA were in good agreement with those obtained by RT-PCR ($r^2 = 0.93$).

10

EXAMPLE 4: Decrease in α -MHC and increase in β -MHC gene expression with aging

The amount of mRNA coding for α -MHC and β -MHC in total mRNA extracted from LVs was measured. The LVs were taken from 15 the hearts of 22 prospective organ donors of varying ages. The organ donors were ultimately rejected for transplantation for non-cardiac reasons, but they had no history of cardiac disease and had normal cardiac function by medical history and echocardiograph or inspection at the time of organ harvest.

20 All procedures were as described in Example 3.

The results are shown in Table 5 below. A significant reduction in percent α -MHC with age was found (*r* value = 0.53, *p* < 0.05). In Table 5, older patients were > 40 years old.

25

Table 5

	<u>Age Group (n)</u>	<u>Age (years)</u>	<u>α-MHC (%)</u>	<u>β-MHC (%)</u>
30	Younger (n=12)	25.8 \pm 2.3	38.2 \pm 5.8	61.8 \pm 5.8
	Older (n=10)	*53.8 \pm 2.0	*19.4 \pm 5.6	*80.6 \pm 5.6

35 **p* < 0.05 versus younger

EXAMPLE 5: Up-regulation of α -MHC and down-regulation of β -MHC by medical treatment

The amount of mRNA coding for α -MHC, β -MHC, β_1 AR, β_2 AR, SRCA, and ANP in total mRNA extracted from endomyocardial biopsies was measured as described in Example 1. The tissues were taken from 29 human subjects diagnosed with idiopathic dilated cardiomyopathy who had completed six months of treatment with either β -adrenergic blockade, a medical treatment which can improve systolic function, or placebo.

10 The β -blocking agents used were either carvedilol or metoprolol.

The results are given in Table 6 below. The subjects are divided into those who experienced improvement in LV function (EF increased by > 5 units) versus those who did not (EF showed no significant change or decreased). All subjects improving ($n=15$) were on β -blockade. Eleven of the 14 subjects not improving were on placebo, and three were on β -blockade. In the subjects showing improved ventricular function, the average LVEF improved from 21-41% and the average RVEF improved from 30-40%. As can be observed from Table 6, the only statistically significant changes exclusively associated with improved left ventricular function are an increase in the amount of α -MHC mRNA and a decrease in the amount β -MHC.

Table 6
(molecules mRNA $\times 10^5$ / μ g total RNA \pm SEM)

Category	Δ LVEF Response	$\Delta\beta_1$ AR	$\Delta\beta_2$ AR	Δ ANP	Δ SRCA	$\Delta\alpha$ -MHC	$\Delta\beta$ -MHC
Improved (n=15)	+18.5 \pm 2.2*#	-0.17 \pm 0.32	+0.22 \pm 0.18	-36.6 \pm 14.2	+5.7 \pm 5.1	+6.8 \pm 3.7*\$	-16.4 \pm 20.6*
Not Improved (n=14)	-1.4 \pm 1.2	-0.25 \pm 0.38	+0.21 \pm 0.75	-36.4 \pm 19\$	+1.8 \pm 7.0	-3.8 \pm 1.5#	+54.8 \pm 26.1\$

* p<0.05 versus not improved.

p<0.05 versus baseline

\$p<0.10 versus baseline

EXAMPLE 6: Effect of Thyroid Hormone on Human Hearts

Contractile responses of isolated right ventricular (RV) trabeculae harvested from transplant recipients with end-stage heart failure (failing) were measured as previously described 5 in Bristow et al., *Circ. Res.*, 59, 297-309 (1986). Briefly, isolated RV trabeculae of uniform size (2 mm \times 8 mm) were mounted in an eight-chamber muscle bath containing Tyrode's solution bubbled with 95% O₂ - 5% CO₂ and paced at 1.0 Hz at 10% above the threshold for initiating contraction. Resting 10 tension placed on the trabeculae was set at the length that produced the maximum degree of systolic contraction, usually \approx 1 gram tension. Tissues were assigned to a treatment group and incubated for 4 or 12 hours with 1 nM triiodothyronine (T3) or vehicle (as control), with bath changes every 30 15 minutes. At the end of the incubation period, full dose-response curves to isoproterenol were performed using 0.5 log unit dose increments between 10⁻⁹ and 10⁻⁴ M. After completion of the isoproterenol dose-response curve and wash-out of isoproterenol, the maximal response to calcium was measured by 20 administering calcium chloride at final concentrations of 2.5, 5, and 10 mM. Tension was recorded as the stimulated tension

minus baseline tension, and the maximum response was taken as the greatest amount of net tension produced at any point in the dose-response curve. Finally, after the contractility measurements were completed, the α -MHC, β -MHC, β -AR, and SRCA gene expression in the trabeculae were measured by quantitative RT-PCR as described in Example 1.

The results of the isoproterenol contractility measurements are presented in Figures 1A (4-hour incubation) and 1B (12-hour incubation). The α -MHC and β -MHC gene expression measurements are presented in Table 7 below. There were no significant differences in adrenergic receptor or SRCA gene expression in the T3 group as compared to the control group.

TABLE 7
Percentage of α - and β -MHC isoform mRNA present
at baseline and following incubation with and without T3

	% α -MHC	% β -MHC
Baseline	10.7 \pm 3.19	89.3 \pm 3.19
+ T3 incubation	24.5 \pm 9.29*	75.5 \pm 9.29*
- T3 incubation	14.6 \pm 6.67	85.4 \pm 6.66

*p<.05 vs. no T3

While various embodiments of the present invention have been described in detail, it is apparent that modifications and adaptations of those embodiments will occur to those skilled in the art. It is to be expressly understood, however, that such modifications and adaptations are within the scope of the present invention, as set forth in the following claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Bristow, Michael R.
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Nakao, Koichi

(ii) TITLE OF INVENTION: "DIAGNOSIS AND TREATMENT OF MYOCARDIAL FAILURE"

(iii) NUMBER OF SEQUENCES: 9

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(F) ZIP: 80203

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.30

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Crook, Wannell M.
(B) REGISTRATION NUMBER: 31,071
(C) REFERENCE/DOCKET NUMBER: 3595-6

(ix) TELECOMMUNICATION INFORMATION:

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(B) TELEFAX: (303) 863-0223

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 21 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ATCAAGGAGC TCACCTACCA G

21

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:2:

CTTTTACGTG CTACTCCTCA C

21

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 20 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:3:

GACCTCGGAC ACATTGTCGA

20

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:4:

ATCAAGGAGC TCACCTACCA GGTGAGGGAGT AGCACGTAAA AG

42

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 42 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:5:

CTTTTACGTG CTACTCCTCA CCTGGTAGGT GAGGCCGGG AT

42

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:6:

ATCAAGGAGC TCACCTACCA GTCGACAATG TGTCCGAGGT C

41

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 41 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:7:

GACCTCGGAC ACATTGTCGA CTGGTAGGTG AGCGCCGGGA T

41

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:8:

AGCAGAACGCG CAACGGAGAG T

21

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:

- (A) LENGTH: 21 base pairs
- (B) TYPE: nucleic acid
- (C) STRANDEDNESS: single
- (D) TOPOLOGY: linear

(ii) MOLECULE TYPE: other nucleic acid
(A) DESCRIPTION: /desc = "primer"

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:9:

TGCTGCACCT TGCGGAACCTT G

21

WE CLAIM:

1. A method of diagnosing myocardial failure in a human comprising:

obtaining a sample of myocardial tissue from a ventricle of the heart of the human;

5 quantitating the expression of α -myosin heavy chain (α -MHC), β -myosin heavy chain (β -MHC), or both in the sample; and

determining by statistical analysis if the expression of α -MHC, β -MHC, or both in the sample is significantly different than their expression in normal human ventricular myocardial

10 tissue.

2. The method of Claim 1 wherein the sample is from the left ventricle of the heart.

3. The method of Claim 1 wherein the expression of α -MHC, β -MHC, or both is quantitated by:

extracting RNA from the tissue;

preparing cDNA from the RNA;

5 amplifying the cDNA coding for α -MHC, β -MHC, or both by polymerase chain reaction (PCR) using primers that hybridize to cDNA coding for α -MHC, β -MHC, or both; and

quantitating the amplified PCR product(s).

4. The method of Claim 3 wherein the primers are labeled to allow for quantitation of the amplified PCR product(s).

5. The method of Claim 3 wherein labeled nucleotides are used during the PCR to allow for quantitation of the amplified PCR product(s).

6. The method of Claim 3 wherein one or more nucleic acid molecules which hybridize to the amplified PCR product(s) is added after the PCR to allow for quantitation of the amplified PCR product(s).

7. The method of Claim 3 wherein the detection of DNA of an expected size allows for quantitation of the amplified PCR product(s).

8. The method of Claim 7 wherein the primers hybridize to both the α -MHC and β -MHC cDNAs, and the method further comprises the step of contacting the amplified PCR products with a restriction enzyme that cleaves one of the products but not the other.

9. The method of Claim 3 further comprising:
providing an internal standard cRNA;
simultaneously preparing cDNA from the internal standard cRNA and the RNA from the sample; and
simultaneously amplifying the cDNA prepared from the internal standard cRNA and the cDNA prepared from the RNA from the sample.

10. The method of Claim 3 wherein the expression of α -MHC and the expression of β -MHC both are determined, and the ratio of the α -MHC expression to β -MHC expression or to total α -MHC and β -MHC expression is calculated.

11. The method of Claim 10 wherein the primers hybridize to both α -MHC and β -MHC cDNAs, and the method further comprises:

contacting the amplified PCR products with a restriction enzyme that cleaves one of the products but not the other;

separating the cleaved and uncleaved PCR products by size; and
quantitating the separated PCR products.

12. A kit for diagnosing myocardial failure in a human comprising a container holding at least one nucleic acid molecule that hybridizes to DNA or RNA coding for α -MHC, β -MHC or both.

13. The kit of Claim 12 wherein the nucleic acid molecule(s) comprise(s) one or more polymerase chain reaction primers.

14. The kit of Claim 12 wherein the one or more primers hybridize to the cDNA coding for α -MHC and to the cDNA coding for β -MHC.

15. The kit of Claim 12 wherein the one or more nucleic acids are labeled.

16. The kit of Claim 13 further comprising a second container holding an internal standard cRNA.

17. A method of treating myocardial failure in a human comprising administering an effective amount of an agent that directly causes an increase in the quantity of α -myosin heavy chain (α -MHC) in the myocardial tissue of the heart

18. The method of Claim 17 wherein the agent is thyroid hormone or an analog thereof.

19. The method of Claim 17 wherein the myocardial tissue is located in a ventricle of the heart.

20. The method of Claim 19 wherein the myocardial tissue is located in the left ventricle.

21. The method of Claim 17 wherein the myocardial failure occurs in a human suffering from heart failure.

22. The method of Claim 17 wherein the myocardial failure occurs in an aging human.

23. The method of Claim 17 wherein the agent is a transgene coding for α -MHC.

24. A method of quantitating the expression of a first protein relative to the expression of a second protein or to the total expression of the first and second proteins comprising:

5 obtaining a sample of cells or tissue expressing the first protein and the second protein;

 extracting RNA from the cells or tissue;

 preparing cDNA from the RNA;

10 amplifying the cDNA coding for the first and second proteins by polymerase chain reaction (PCR) using primers that hybridize to cDNA coding for the first protein, the second protein or both; and

 quantitating the amplified PCR products.

25. The method of Claim 24 wherein one pair of primers is used, and the primers hybridize to both the cDNA coding for the first protein and the cDNA coding for the second protein.

26. The method of Claim 25 further comprising:

contacting the amplified PCR products with a restriction enzyme that cleaves one of the products but not the other;

separating the cleaved and uncleaved PCR products by
5 size; and

quantitating the separated PCR products.

ABSTRACT

The invention provides a method of diagnosing myocardial failure in a human. The method comprises: obtaining a sample of myocardial tissue from a ventricle of the heart of the
5 human; quantitating the expression of α -myosin heavy chain (α -MHC), β -myosin heavy chain (β -MHC), or both in the sample; and determining by statistical analysis if the expression of α -MHC, β -MHC, or both in the sample is significantly different than their expression in normal human ventricular myocardial
10 tissue.

The invention also provides a kit for diagnosing myocardial failure in a human. The kit comprises a container holding at least one nucleic acid molecule that hybridizes to DNA or RNA coding for α -MHC, β -MHC or both.

15 The invention further provides a method of treating myocardial failure in a human. The method comprises administering an effective amount of an agent that directly causes an increase in the quantity of α -MHC in the myocardial tissue of the heart.

20 Finally, the invention provides a method of quantitating the expression of a first protein relative to the expression of a second protein or to the total expression of the first and second proteins. The method comprises obtaining a sample of cells or tissue expressing the first protein and the second
25 protein, extracting RNA from the cells or tissue, preparing cDNA from the RNA, amplifying the cDNA coding for the first and second proteins by polymerase chain reaction using primers that hybridize to cDNA coding for the first protein, the second protein or both, and quantitating the amplified PCR
30 products.

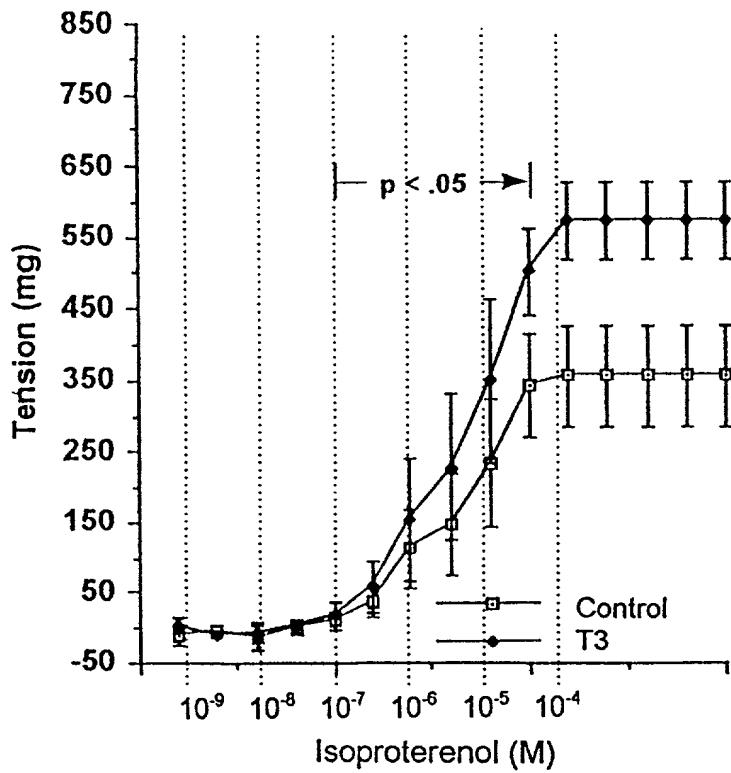


Fig. 1A

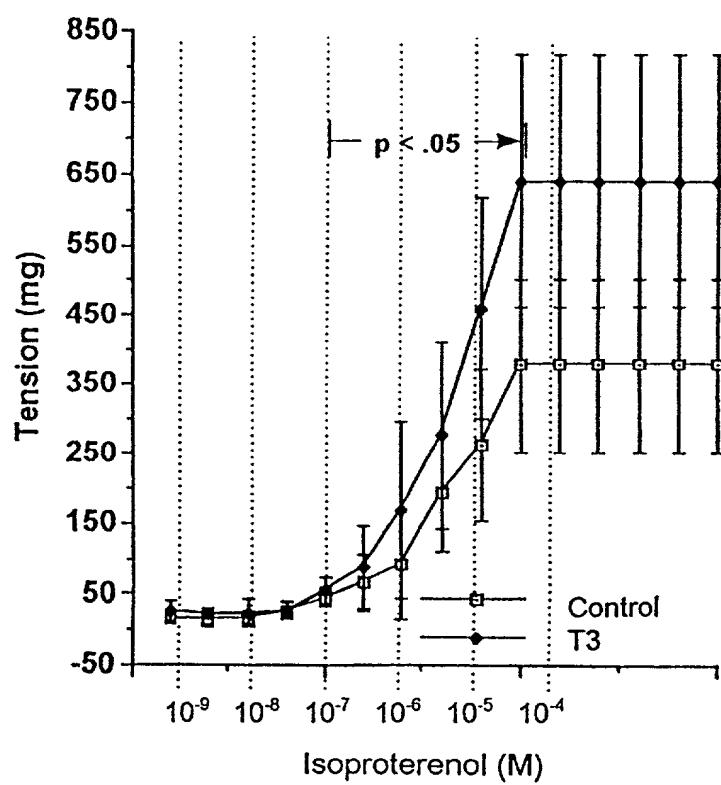


Fig. 1B

RULE 63 (37 CFR § 1.63)
DECLARATION FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled "DIAGNOSIS AND TREATMENT OF MYOCARDIAL FAILURE," the specification of which has been prepared and filed on January 30, 1998, receiving Serial No. 09/016,075, and further identified as Attorney File No. 3595-6.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability in accordance with 37 CFR 1.56(a) and (b) as set forth on the attached sheet indicated Page 3 hereof and which I have read.

I hereby claim priority benefits under 35 U.S.C. 119(e) of any provisional application(s) for patent listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information material to patentability in accordance with 37 CFR 1.56(a) and (b) which occurred between the filing date(s) of the prior application(s) and the national or PCT international filing date of this application:

<u>Application Serial No.</u>	<u>Filing Date</u>	<u>Status</u>
60/036,987	1/30/97	expired
60/038,911	2/26/97	expired

I hereby claim the benefit under 35 U.S.C. 120/365 of all United States and PCT international applications listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information material to patentability in accordance with 37 CFR 1.56(a) and (b) which occurred between the filing date(s) of the prior application(s) and the national or PCT international filing date of this application:

Application Serial No. Filing Date Status: patented, pending, abandoned

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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Residence: 135-2 chikami-cho III-205
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*Complete Post Office Address in full if different from Residence, otherwise indicate that the Post Office Address is "Same as Residence."

37 CFR §1.56(a) and (b)
DUTY TO DISCLOSE INFORMATION MATERIAL
TO PATENTABILITY

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is cancelled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

(1) prior art cited in search reports of a foreign patent office in a counterpart application, and

(2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

(1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or

(2) It refutes, or is inconsistent with, a position the applicant takes in:

(i) Opposing an argument of unpatentability relied on by the Office, or

(ii) Asserting an argument of a patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.*

*Note, 37 CFR §1.97(h) states: "The filing of an information disclosure statement shall not be construed to be an admission that the information cited in the statement is, or is considered to be, material to patentability as defined in §1.56(b)."

RULE 63 (37 CFR § 1.63)
DECLARATION FOR PATENT APPLICATION
IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

As a below named inventor, I hereby declare that my residence, post office address and citizenship are as stated below next to my name, and I believe I am an original, first and joint inventor of the subject matter which is claimed and for which a patent is sought on the invention entitled "DIAGNOSIS AND TREATMENT OF MYOCARDIAL FAILURE," the specification of which has been prepared and filed on January 30, 1998, receiving Serial No. 09/016,075, and further identified as Attorney File No. 3595-6.

I hereby state that I have reviewed and understand the contents of the above-identified specification, including the claims, as amended by any amendment referred to above. I acknowledge the duty to disclose information which is material to patentability in accordance with 37 CFR 1.56(a) and (b) as set forth on the attached sheet indicated Page 3 hereof and which I have read.

I hereby claim priority benefits under 35 U.S.C. 119(e) of any provisional application(s) for patent listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information material to patentability in accordance with 37 CFR 1.56(a) and (b) which occurred between the filing date(s) of the prior application(s) and the national or PCT international filing date of this application:

<u>Application Serial No.</u>	<u>Filing Date</u>	<u>Status</u>
60/036,987	1/30/97	expired
60/038,911	2/26/97	expired

I hereby claim the benefit under 35 U.S.C. 120/365 of all United States and PCT international applications listed below and, insofar as the subject matter of each of the claims of this application is not disclosed in such prior applications in the manner provided by the first paragraph of 35 U.S.C. 112, I acknowledge the duty to disclose information material to patentability in accordance with 37 CFR 1.56(a) and (b) which occurred between the filing date(s) of the prior application(s) and the national or PCT international filing date of this application:

Application Serial No. Filing Date Status: patented, pending, abandoned

I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

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37 CFR §1.56(a) and (b)
DUTY TO DISCLOSE INFORMATION MATERIAL
TO PATENTABILITY

(a) A patent by its very nature is affected with a public interest. The public interest is best served, and the most effective patent examination occurs when, at the time an application is being examined, the Office is aware of and evaluates the teachings of all information material to patentability. Each individual associated with the filing and prosecution of a patent application has a duty of candor and good faith in dealing with the Office, which includes a duty to disclose to the Office all information known to that individual to be material to patentability as defined in this section. The duty to disclose information exists with respect to each pending claim until the claim is cancelled or withdrawn from consideration, or the application becomes abandoned. Information material to the patentability of a claim that is cancelled or withdrawn from consideration need not be submitted if the information is not material to the patentability of any claim remaining under consideration in the application. There is no duty to submit information which is not material to the patentability of any existing claim. The duty to disclose all information known to be material to patentability is deemed to be satisfied if all information known to be material to patentability of any claim issued in a patent was cited by the Office or submitted to the Office in the manner prescribed by §§1.97(b)-(d) and 1.98. However, no patent will be granted on an application in connection with which fraud on the Office was practiced or attempted or the duty of disclosure was violated through bad faith or intentional misconduct. The Office encourages applicants to carefully examine:

(1) prior art cited in search reports of a foreign patent office in a counterpart application, and

(2) the closest information over which individuals associated with the filing or prosecution of a patent application believe any pending claim patentably defines, to make sure that any material information contained therein is disclosed to the Office.

(b) Under this section, information is material to patentability when it is not cumulative to information already of record or being made of record in the application, and

(1) It establishes, by itself or in combination with other information, a prima facie case of unpatentability of a claim; or

(2) It refutes, or is inconsistent with, a position the applicant takes in:

- (i) Opposing an argument of unpatentability relied on by the Office, or
- (ii) Asserting an argument of a patentability.

A prima facie case of unpatentability is established when the information compels a conclusion that a claim is unpatentable under the preponderance of evidence, burden-of-proof standard, giving each term in the claim its broadest reasonable construction consistent with the specification, and before any consideration is given to evidence which may be submitted in an attempt to establish a contrary conclusion of patentability.*

*Note, 37 CFR §1.97(h) states: "The filing of an information disclosure statement shall not be construed to be an admission that the information cited in the statement is, or is considered to be, material to patentability as defined in §1.56(b)."